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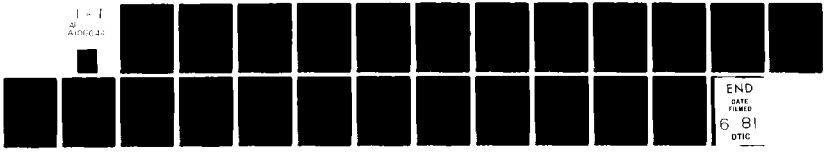

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A general review of postulated *Legionella pneumophila* toxins is presented. A common *Legionella* toxin is identified in not only *L. pneumophila* but *L. gormanii*, *L. dumoffii* and *bozemanii*. The role of this toxin in providing cross protection against heterologous challenge is demonstrated.

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LEGIONELLA TOXIN

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In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.

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Figures: 4

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Abstract — The presence of a Legionella pneumophila toxin has been clinically suspected since 1978. A common 3,400 molecular weight protein has been found in the cell free extracts of sonicated L. pneumophila organisms serotype 1, as well as L. dumoffii, L. bozemanii, L. micdadei, and L. gormanii organisms, although they are genetically distinct by DNA homology studies. This toxic entity is lethal for macrophages and suppresses the chemiluminescent activity of human polymorphonuclear leucocytes. Since both these cell types are of primary importance in limiting bacterial disease, the common Legionella toxin may play a pivotal role in the establishment of Legionnaire and Legionnaire-like infections. The common antigenicity provides cross-protection in animals immunized with toxin derived from one Legionella species and then cross-challenged with either viable organisms or toxins from a genetically distinct Legionella species. The mechanisms of actions are still being defined.

1. INTRODUCTION

Unlike the standard introduction for the review of well recognized microbial toxins which may have been studied for decades, this introduction will first attempt to orient the reader with our present understanding about the spectrum of disease caused by infection with the Legionella organisms and then present the early rationale for postulating the existence of a toxin or toxins. There have been a variety of suspected bacterial toxic components. We are in the present position of evaluating the contributory role of each.

At the outset the reader should understand that the term Legionella pneumophila applies to the causative agent of the fatal outbreak of pneumonia that occurred among American Legion conventioneers in Philadelphia in the summer of 1976. In the mid-1980s other gram-negative organisms genetically distinct by DNA homology studies but phenotypically similar to L. pneumophila were given the genus name Legionella for "operational purposes" by Brenner et al. (1980) and this will be developed in greater detail later.

2. THE "NEW PNEUMONIA" AGENTS: CLINICAL ASPECTS THAT LED TO THE CONCEPT OF TOXIN

It is apparent that laboratories once primarily concerned with the recognition of the etiologic agent of Legionnaires' disease must now by necessity be able to recognize and study the ever expanding spectrum of "new pneumonia" agents. These "new pneumonias" have been clinical entities for years. As Weinstein (1980) has noted, the only new feature is the identification of their specific etiologic agents, a group of gram-negative bacilli. A great debt is owed to both Fraser et al. (1977) and McDade et al. (1977) who were the first to isolate and identify the causative

agent of Legionnaires' disease only six months after the 1976 Philadelphia outbreak. Their pioneering discovery and demonstration of the relationship between this previously unrecognized gram-negative bacteria and the illness caused by it have expanded medical horizons. Two clinical entities are now recognized as being caused by L. pneumophila. The first is the intensely studied pneumonic form of the illness called Legionnaires' disease which may involve patients ranging from 3 to 82 years of age. The initial symptoms are nondescriptive headaches, myalgias, and general malaise. In one or two days there is a rapid temperature rise associated with chills. A moderate nonproductive cough is frequently present and the symptoms progress to include chest pain, abdominal pain, vomiting and mental confusion. Diarrhea is seen in approximately one-sixth of the patients and may precede other symptoms. The clinical course of this rapidly progressive fulminant pneumonia can be followed by chest X-rays that initially show patchy infiltrates that may have an interstitial or consolidated appearance; these typically progress to a unilateral or bilateral nodular consolidation. Laboratory data may reveal both hepatic and renal compromise. Death is associated with respiratory failure or shock.

The second form of recognized legionellosis, as the collective term for diseases caused by L. pneumophila agents has come to be termed, "Pontiac Fever". In July and early August 1968 an epidemic of acute febrile illness occurred which affected 144 people including 95% of the employee staff in a County Health Department building in Pontiac, Michigan. Glick et al. (1978) described this syndrome as a relatively uniform self-limiting illness which sometimes began quite abruptly but more often was marked by progressive malaise, diffuse myalgias, and headaches. Usually the complete syndrome appeared within 6 hr. Localized

muscle pains, dizziness, cough, nausea and mental confusion were common. Diarrhea was reported in 15% of the patients. Respiratory symptoms were not significant although 57% complained of a dry cough. This acute nonpulmonic form of legionellosis tended to last 2-5 days and there have been no reported deaths.

Although clinical attention was focused primarily on the pulmonic component of Legionnaires' disease, it was the multisystem involvement of lungs, kidneys and central nervous system in certain Legionnaires' disease patients that led Friedman (1978) to postulate a bacterial toxin. At the same time and for an entirely different reason Winn et al. (1978), also postulated that a bacterial toxin might be elaborated by the Legionella organism to explain the histopathologic findings of lysis of inflammatory exudate cells and the infarct-like necrosis seen in alveolar walls in lung tissue obtained from a study of 14 patients who had died in the 1977 Vermont outbreak. Fraser et al. (1979) noted that what determined whether L. pneumophila causes Legionnaires' disease or Pontiac fever is entirely unknown. He suggested that Pontiac fever might be the result of a large dose of nontoxigenic organisms. At the time he made that suggestion, no L. pneumophila toxins were recognized. In early 1979 there was only one known serotype of L. pneumophila. At the time of this writing there are six L. pneumophila serotypes which can be shown to be genetically related by DNA homology procedures. It is precisely this coupling of new technologic advances with heightened clinical awareness that led to an unprecedented explosion of information about previously unrecognized gram-negative bacteria which can cause both pulmonic and nonpulmonic illness. In addition to the increase in the numbers of known serotypes of L. pneumophila there has also been the discovery and rediscovery of other gram-negative Legionella-like organisms capable of causing human disease. Pasculle et al. (1980) noted

that certain of the newly recognized gram-negative bacteria are serologically and genetically distinct from L. pneumophila, but phenotypically resemble this organism in the type of pneumonic illness they cause, in growth requirements and in abundance of cellular branched chain fatty acids. Brenner et al. (1980) offered a tentative solution in classifying the newly recognized pneumonia agents. While recognizing that ideally a genus should contain a group of genetically and phenotypically related species, when both criteria cannot be met, phenotypic relatedness should take precedence to ensure that the genus designation is of practical value. Under this convention in addition to L. pneumophila, Brenner has suggested the schema in Table 1.

3. POTENTIAL CANDIDATE TOXINS

Clinical speculations about the pathogenesis of Legionnaires' disease and the possibility of toxins preceded the offering of potential toxin candidates by a different group of scientists. The first classic candidate to have been considered in this gram-negative infection was of course the endotoxin lipopolysaccharide (LPS). Fumarola (1978) published a short letter stating that L. pneumophila organisms, then termed Legionnaires' disease agent, were limulus lysate-positive. Since then Wong et al. (1979) have convincingly demonstrated that while indeed there is limulus lysate gelating activity, the "endotoxicity" detected by in vitro and in vivo biological assays seemed to be different from the classic endotoxicity associated with gram-negative organisms. With endotoxins from Salmonella, Klebsiella, Escherichia, and other gram-negative species, the limulus lysate test is 10-20 times more sensitive than the rabbit pyrogen test (Wong et al., 1977; Ronneberger, 1977). There was a greater than 1000-fold difference between these two tests

observed with L. pneumophila. In addition to their low pyrogenicity, L. pneumophila organisms were also found to be very weak in inducing heparin-precipitable protein and Schwartzman reactions in rabbits. Endotoxicity was of a low order as measured by Dactinomycin potentiation or polymyxin B inhibition. The author (Hedlund, unpublished data) has given as much as 15 µg of 2-keto-3-deoxyoctonate (KDO) containing L. pneumophila LPS to AKR/J mice without lethal effect.

Baine et al. (1979a) was the first to suggest that an enzymatic exotoxin-like activity was associated with L. pneumophila organisms based on hemolysis of guinea pig red blood cells incubated in the presence of allantoic fluid in which L. pneumophila had grown. Later Baine et al. (1979b) also noted hemolytic activity in plasma and urine from L. pneumophila-infected rabbits. Unfortunately a direct connection could not be made and Baine cautioned that indeed the hemolytic activity in the filtrate of allantoic fluid might be due to the presence of agents other than bacterial hemolysins, inasmuch as normal allantoic fluid spontaneously developed hemolytic activity in the absence of contact with a healthy embryo.

Muller (1980) has shown that at least four strains of L. pneumophila could degrade α_1-acid glycoprotein, α_1-chymotrysin, ß-lipoprotein, ß-IgE-globulin and α_2-glycoprotein. He suggested that the pathogenic action of L. pneumophila might be due to the proteolytic activities, since the degraded proteins either belonged to the acute-phase protein group or, as is the case with ß-lipoproteins was involved with nonspecific resistance against infection.

Friedman et al. (1980) demonstrated that culture filtrates of L. pneumophila were cytotoxic for Chinese hamster ovary cells. The cytotoxin was methanol-soluble, heat-stable and stable from pH 5-8. The

cytotoxin was sensitive to pronase and papain and insensitive to trypsin. Friedman suggested that it was a small polypeptide, but large enough to be retained by a dialysis membrane with a molecular weight cut-off of 1,000.

In contrast to a cytotoxin released by L. pneumophila under certain culture conditions, Katz et al. (1980) reported that macrophage monolayers obtained from guinea pigs, mice or rats were rapidly lysed on incubation with 10 virulent L. pneumophila organisms per cell. Washed L. pneumophila caused destruction of 50% of mouse peritoneal macrophage monolayers after 4 hr incubation, when assessed by trypan blue exclusion, along with light and electron microscopy. They found that opsonization enhanced by specific antisera increased monolayer toxicity to greater than 95% in 4 hr. Phagocytic inhibition by cytochalasin D and lidocaine caused a marked decrease in monolayer lysis. Supernatants of virulent L. pneumophila suspensions, ultraviolet or heat-killed L. pneumophila were not toxic to macrophage monolayers. The rapid time course suggested that while viable organisms were necessary for macrophage destruction, intracellular replication was not. Katz therefore felt that the data were not consistent with the action of a preformed soluble toxin, but suggested intracellular release of a toxin as a basis for the observed macrophage toxicity.

Hedlund et al. (1979) initially demonstrated that L. pneumophila were lethal for AKR/J mice. Later Hedlund and Larson (1981) showed that cell-free sonicates of the same organism were also lethal when injected i.p. into AKR/J mice. Acid partition of this crude toxin preparation followed by gel filtration and preparative isotachophoresis of the resultant supernatant material yielded a 3,400 molecular weight toxin. At the time when these preliminary experiments on L. pneumophila toxin

purification were being done, the other new genetically distinct Legionella agents were being recognized. It now was a simple matter to extend these findings and techniques to the new members of the Legionella family. Representatives of all the known Legionella species were kindly provided by both Dr. Pasculle of the University of Pittsburgh School of Medicine, Pennsylvania, and the Center for Disease Control in Atlanta, Georgia. These organisms were subjected to the identical cultural, harvesting and toxin separation procedures that were used on L. pneumophila and previously reported (Hedlund and Larson, 1981). We drew attention to the fact that cell-free acid supernatants of sonicated L. pneumophila and the genetically distinct L. micdadei (Pittsburgh pneumonia agent) were lethal for AKR/J mice. We reported that both contained 3,400 molecular weight proteins which were antigenically identical. These extended studies demonstrate that L. pneumophila serotype 1 shares a common toxic low molecular weight antigen with all other identified genetically distinct Legionella species (Fig. 1).

Attempts were made to find in vitro correlates to the AKR/J animal lethality studies that might shed some light on pathogenic mechanisms. New Zealand BWJM mouse macrophages were grown for 48 hr in culture flasks containing Eagles Minimal Essential Medium/Nonessential amino acids medium with 10% fetal calf serum, 1% penicillin-streptomycin and 1% sodium pyruvate; incubation was at 37°C. Cells were removed from the growth surface by gently rolling glass beads across them. The suspension had a cell count of 6.4×10^5 cells/ml with a viability of 95%. The control sample contained 1.0 ml of cell suspension and 0.5 ml of preparative isotachophoresis elution buffer (H_3PO_4 Tris, pH 7.04). The treated sample contained 1.0 ml of an identical cell suspension and 0.5 ml of the isolated isotachophoretic peak derived from L. pneumophila

organisms. The samples were incubated in tightly capped 5-ml Falcon plastic tubes at 37°C. Viabilities were determined by the standard trypan blue exclusion method. The results are shown in Fig. 2. It is apparent that within 4 hr the number of viable toxin-treated macrophages dropped to less than one-half of the control levels.

Another demonstration of Legionella's toxic impact on cells normally involved in antimicrobial defense is provided by chemiluminescence (CL) studies. Basically polymorphonuclear leukocytes (PMN) emit CL after phagocytosis of certain opsonized particles, like bacteria. Light emission can be detected and quantitated in a liquid scintillation counter and appears to result from the ground state of electronically excited carbonyl groups, thought to be generated during singlet oxygen-mediated oxidation of the phagocytized substrate; one of the earliest studies by Stevens and Young (1976) demonstrated a correlation between resistance of certain strains of E. coli to opsonization and decreased in vitro killing, oxygen consumption, visual phagocytosis and CL responses of human granulocytes. Grebner et al. (1976) demonstrated parallel relationships between phagocytosis and CL under a variety of conditions designed to alter opsonization of bacteria. This led the authors to conclude that the biochemical processes controlling phagocytosis and CL may be closely related or interdependent. In addition to phagocytosis, Allen et al. (1974) also established the relationship of CL measurements to the microbicidal activity of PMN. As noted by Trush et al. (1978) the CL response of phagocytic cells is dependent on cell metabolism and the measurement of CL represents a potentially useful index to assess the effects of pharmacologically toxic agents on phagocytic cells. To study the effects of Legionella toxin on CL, preparative isotachophore peaks obtained from L. pneumophila

(Washington strain, serotype 1) and L. micdadei were passed over an anion exchange column and two separate peaks were obtained (Fig. 3).

Similar bimodal peaks could be obtained from the other Legionella species. CL techniques previously described by McCarthy et al. (1980), but specifically adapted to use human PMN as well as rat PMN were used to test the toxicity of the Legionella toxins. The results are shown in Fig. 4. It can be clearly seen that the second peak consistently inhibits the CL activity of the PMN.

The next question that arose was how readily can these "in vitro" similarities of antigenicity, molecular weight, toxic functions be translated into in vivo models? Could animals immunized with L. pneumophila be protected against a lethal challenge from genetically distinct L. micdadei? The following set of experiments were performed using the Washington strain serotype I, L. pneumophila and L. micdadei (Pittsburgh pneumonia agent) obtained from Dr. Pasculle. DNA homology studies were performed to document their genetic distinctness (Pasculle et al., 1980). AKR/J mice were inoculated with sublethal aliquots of acid supernatant material from both species of Legionella obtained by methods previously described (Hedlund and Larson, 1981), and then boosted 28 days later. After 10 days they were challenged with either a lethal dose of viable L. pneumophila or L. micdadei or a lethal dose of acid supernatant material from the respective organisms. Nonimmunized mice were included for the appropriate lethal challenge controls. Results are shown in Table 2. Animals immunized with L. pneumophila acid supernatant and challenged with a lethal inoculum of viable L. pneumophila or L. micdadei organisms are protected, as are animals given a lethal inoculation of L. pneumophila or L. micdadei acid supernatant.

This cross-protection by a previously demonstrated single shared antigen was also confirmed when animals were immunized with L. micdadei acid supernatant and challenged with a lethal concentration of either viable L. micdadei or L. pneumophila organisms or their acid supernatants. Studies using L. pneumophila preparative tachophore peaks which contain the single shared, common antigen were used to protect animals with the same effectiveness as the acid supernatant preparations, although in this low molecular weight form they are probably less efficient as an antigen and harder to obtain.

4. SUMMARY

This is a chapter on Legionella toxins that is still being defined. It originally started as a review based just on L. pneumophila at a time before the operational designations L. micdadei, L. dumoffii, L. gormanii and L. bozemanii were even suggested. The role of Legionella lipopolysaccharides with their limited expression of classic endotoxicity as well as the role of the various enzyme components found are still being evaluated, but appear to be of limited impact.

At present there are no amino acid analysis studies, no receptor site studies, and no molecular mechanism of action analyses, but we still are in early times. What we do know is that there is a low molecular weight protein of approximately 3,400 daltons which can be isolated after disruption of the intact organism. This single common toxic moiety is shared by L. pneumophila serotype 1 and L. micdadei, L. dumoffii, L. bozemanii and L. gormanii. The toxin is capable of killing mouse macrophages and suppressing the oxidative metabolism of rat and

human polymorphonuclear leukocytes. This induced depressed metabolic state has been associated with impaired phagocytic and bactericidal activity.

Whether or not this low molecular weight protein is an intact toxin or simply a biologically active piece of the parent molecule is not known at this time. The low molecular weight supernatant cytotoxin described by Friedman et al. (1980) and the intracellular cytotoxin of Katz et al. (1980) which have been associated in L. pneumophila may well be related to the common Legionella toxin described by Hedlund and Larson (1981) and expanded upon here.

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TABLE 1. Proposed New Designation for *Legionella* Species

New Name	Old Name
L. pneumophila	Legionnaires' disease organism, OLDA
L. bozemanii	WIGA, MI 15
L. dumoffii	NY 23, TEX-KL
L. micdadei	Tatlock, HEBA, Pittsburgh Pneumonia Agent
L. gormanii	

TABLE 2. Effect of Immunization of AKR/J Mice with *Legionella* Toxin

Challenged	Survivors/Total		
	Immune		Nonimmune
	L. pneumophila	*L. micdadei*	
1. *L. pneumophila* (as[a])	6/6		0/6
2. *L. pneumophila* (whole)	6/6		0/6
3. *L. micdadei* (whole)	6/6		1/6
1. *L. micdadei* (as)		6/6	0/6
2. *L. micdadei* (whole)		6/6	0/6
3. *L. pneumophila* (whole)		4/6	0/6

[a]Acid supernatant.

FIGURE LEGENDS

FIG. 1. The common Legionella toxin demonstrated by a line of identity connecting various Legionella species sources. A. L. pneumophila; B. L. micdadei; C. L. bozemanii; D. L. dumoffii; and E. L. gormanii; F. E. coli acid supernatant. Center well contains antibodies from L. pneumophila-immunized goat previously absorbed with E. coli/pseudomonas acid supernatant material.

FIG. 2. Survival of mouse macrophages. Cross-hatched areas refer to L. pneumophila toxin challenged cells.

FIG. 3. Anion exchange separation of Legionella pneumophila of preparative tachophore peak. Only the second peak affected chemiluminescence.

FIG. 4. Effect of Legionella toxin on PMN chemiluminescence. Column A is the bovine serum albumin control. B and C are 0.8-μg aliquots of the first and second anion exchange L. micdadei peaks. D and E are 0.8-μg aliquots of similar L. pneumophila anion exchange peaks.

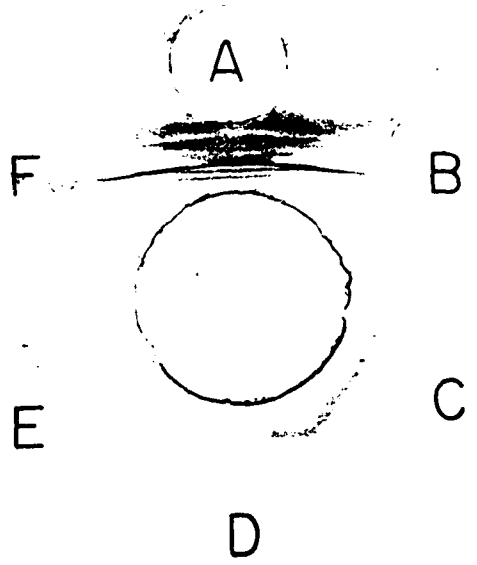
A
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FIG. 1

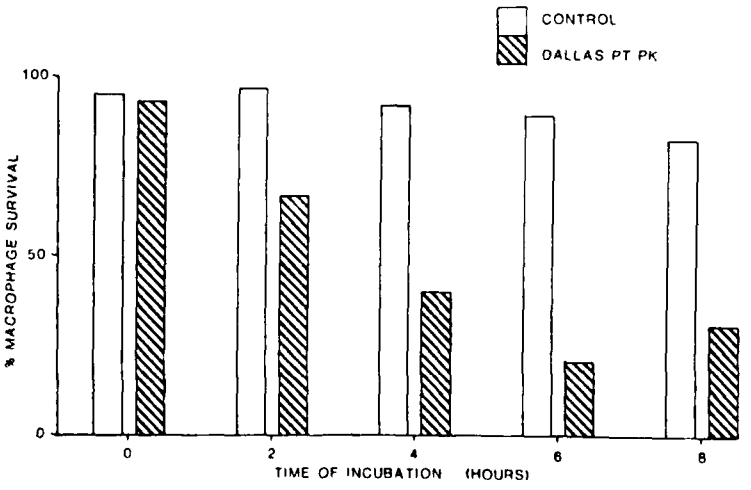
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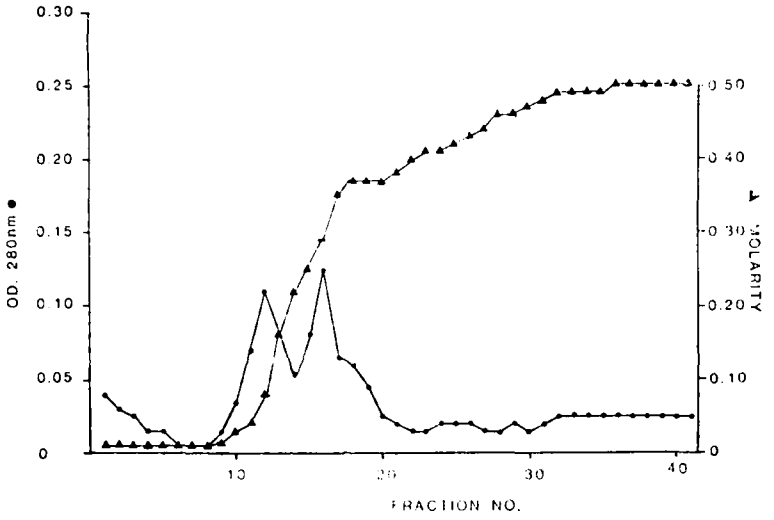
FIG. 2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0.25
0.20
0.15
0.10
0.05
0
OD. 280nm
0.50
0.40
0.30
0.20
0.10
0
10
20
30
40
FRACTION NO.

FIG. 3

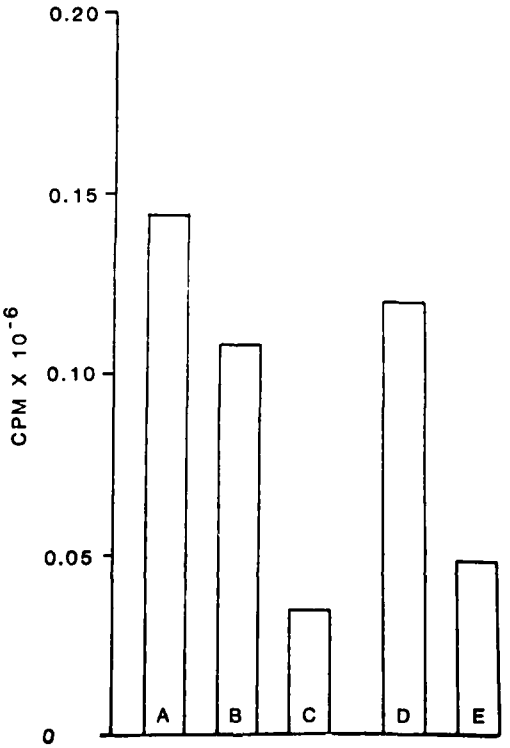
0.20
0.15
0.10
0.05
0
CPM X 10⁻⁶
A
B
C
D
E

FIG. 4